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Minireview

Na^+-Translocating NADH-Quinone Reductase of Marine and Halophilic Bacteria

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The respiratory chain of marine and moderately halophilic bacteria requires Na^+ for maximum activity, and the site of Na^+-dependent activation is located in the NADH-quinone reductase segment. The Na^+-dependent NADH-quinone reductase purified from marine bacterium *Vibrio alginolyticus* is composed of three subunits, α, β, and γ, with apparent M_r of 52, 46, and 32 kDa, respectively. The FAD-containing β-subunit reacts with NADH and reduces ubiquinone-1 (Q-1) by a one-electron transfer pathway to produce ubisemiquinones. In the presence of the FMN-containing α-subunit and the γ-subunit, Q-1 is converted to ubiquinol-1 without the accumulation of free radicals. The reaction catalyzed by the α-subunit is strictly dependent on Na^+ and is strongly inhibited by 2-*n*-heptyl-4-hydroxyquinoline N-oxide (HQNO), which is tightly coupled to the electrogenic extrusion of Na^+. A similar type of Na^+-translocating NADH-quinone reductase is widely distributed among marine and moderately halophilic bacteria. The respiratory chain of *V. alginolyticus* contains another NADH-quinone reductase which is Na^+ independent and has no energy-transducing capacity. These two types of NADH-quinone reductase are quite different with respect to their mode of quinone reduction and their sensitivity toward NADH preincubation.

KEY WORDS: Na^+ transport; NADH-quinone reductase; Na^+ pump; respiratory chain; flavoprotein; marine bacteria; halophilic bacteria.

INTRODUCTION

Marine and halophilic bacteria require Na^+ for optimal growth (MacLeod, 1965; Reichelt and Baumann, 1974; Kushner, 1978). A first clue as to the role of Na^+ in the growth of marine bacteria was the observation that Na^+ was required for the active uptake of nutrients (Drapeau and MacLeod, 1963; Wong *et al.*, 1969). Using α-aminoisobutyric acid (AIB) as a nonmetabolizable amino acid analog, the transmembrane electrochemical potential gradient of Na^+ ($\Delta\tilde{\mu}_{Na^+}$) was shown to be a direct driving force for AIB uptake (Tokuda *et al.*, 1982). Indeed, all ten independent amino acid uptake systems of the marine bacterium *Vibrio alginolyticus*, identified by competition experiments, are absolutely dependent on Na^+ (Unemoto *et al.*, 1990); the active uptake of sucrose is also driven by $\Delta\tilde{\mu}_{Na^+}$ (Kakinuma and Unemoto, 1985). Undoubtedly, Na^+ plays an essential role for the active uptake of nutrients in the marine and halophilic bacteria.

An extremely halophilic *Halobacterium halobium* (Lanyi, 1979) and alkalophilic bacilli (Kitada and Horikoshi, 1977; Krulwich, 1986) also utilize $\Delta\tilde{\mu}_{Na^+}$ for the active uptake of nutrients. In these organisms, the $\Delta\tilde{\mu}_{Na^+}$ is generated by a secondary Na^+/H^+ antiporter, which is driven by the electrochemical potential gradient of H^+ ($\Delta\tilde{\mu}_{H^+}$). In contrast, the marine *V. alginolyticus* was found to have a respiration-driven primary Na^+ pump in addition to the $\Delta\tilde{\mu}_{H^+}$-driven Na^+/H^+ antiporter (Tokuda and Unemoto, 1981, 1982). The electrogenic Na^+ extrusion is directly coupled to the Na^+-dependent NADH-quinone reductase segment of the respiratory chain (Tokuda

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and Unemoto, 1984; Hayashi and Unemoto, 1984, 1986, 1987).

Halophilic bacteria are divided into three categories based on their salt requirments for optimal growth (Kushner, 1978). Marine bacteria belong to the slight halohile group which grows best in media containing 0.2–0.5 M NaCl; moderate halophiles grow best in 0.5–2.5 M NaCl; and extreme halophiles in 2.5–5.2 M NaCl. Extreme halophiles are classified into archaebacteria which have unique light-driven H^+-pumping bacteriorhodopsin and Cl^--pumping halorhodopsin (Schobert and Lanyi, 1982), but the primary Na^+ pump has never been reported in these organisms. As compared with marine bacteria, moderate halophiles are able to grow in a wide range of NaCl concentrations and are highly adapted to salt environments. The respiratory chains of moderate halophiles from diverse origins were examined, and the Gram-negative moderate halophiles were found to have a Na^+-translocating NADH-quinone reductase which is very similar to that of marine bacteria (Unemoto *et al.*, 1992b). Therefore, the presence of Na^+-translocating NADH-quinone reductase is a common feature among marine and moderately halophilic bacteria (Unemoto and Hayashi, 1989). This paper reviews the enzymatic properties of Na^+-translocating NADH-quinone reductase and its distribution in the marine and moderately halophilic bacteria.

Na^+-TRANSLOCATING NADH-QUINONE REDUCTASE OF *V. ALGINOLYTICUS*

Salt modifications of enzymes from the marine bacterium *V. alginolyticus* have been studied in detail; periplasmic and membrane-bound phosphohydrolases were found to require salt for activity (Unemoto *et al.*, 1974). However, these enzymes are modified by anions, and no requirement for specific monovalent cations is detected. Later, the membrane-bound NADH oxidase was found to be specifically activated by Na^+ (Unemoto *et al.*, 1977). Other cations such as Li^+, K^+, Rb^+, Cs^+, Mg^{2+}, and Ca^{2+} are ineffective as replacements for Na^+. Although the requirement for Na^+ is greatly reduced in the presence of 400 mM K^+ or 10 mM Mg^{2+}, Na^+ is always required for the maximum activation of NADH oxidase (Unemoto *et al.*, 1977). The respiratory chain of *V. alginolyticus* is composed of ubiquinone (Q), menaquinone (MK), and cytochromes *b*, *c*, *d*, and *o* (Unemoto and Hayashi, 1979). NADH dehydrogenase activity as measured with menadione or ferricyanide as an electron acceptor and quinol oxidase activity as measured with duroquinol, ubiquinol-1, or tetramethyl-*p*-phenylenediamine as an electron donor shows no dependence on Na^+. Conversely, the reduction of Q-1 to ubiquinol-1 by NADH (NADH-Q-1 reductase) requires Na^+ for maximum activity. The reduction by NADH of Q-10 and MK-4 reincorporated into the quinone-depleted membranes is also strictly dependent on Na^+. The cooperative effect of K^+ with Na^+ observed in NADH oxidas is also detected in this reaction. Therefore, it was concluded that the site of Na^+-dependent activation in the respiratory chain of *V. alginolyticus* resides in the NADH-quinone reductase segment of the respiratory chain (Unemoto and Hayashi, 1979).

In 1981, the marine *V. alginolyticus* was found to generate membrane potential ($\Delta\Psi$) at alkaline pH even in the presence of a protonophore, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) (Tokuda and Unemoto, 1981). The generation of a CCCP-resistant $\Delta\Psi$ is strictly dependent on respiration and Na^+ is actively extruded from the cells at pH 8.5 in the presence of CCCP (Tokuda and Unemoto, 1982). Mutants defective in the respiration-driven Na^+ pump were isolated (Tokuda, 1983), and detailed studies on the respiratory chain of these mutants revealed that the respiration-driven Na^+ pump is tightly coupled to the reaction catalyzed by Na^+-dependent NADH-quinone reductase (Tokuda and Unemoto, 1984). Although the mutants were devoid of Na^+-dependent NADH-quinone reductase, the membranes from these mutants catalyzed the reduction of Q-1 to ubiquinol-1. This reaction was found to be catalyzed by a Na^+-independent NADH-quinone reductase (Tokuda and Unemoto, 1984). Thus, there are two different types of NADH-quinone reductase: one is Na^+-dependent (designated NQR-1) and the other is Na^+-independent (designated NQR-2). Both NQR-1 and NQR-2 activities could be detected in the membranes from wild-type cells. Recently, NQR-2 was purified from *V. alginolyticus* (Hayashi *et al.*, 1992). The enzymatic properties of NQR-2 will be described in comparison with that of NQR-1.

SUBUNIT STRUCTURE AND ELECTRON TRANSFER PATHWAY

The Na^+-dependent NADH-quinone reductase (NQR-1) of *V. alginolyticus* was extracted from the

Table I. Subunit Composition, Flavin Content, and Enzyme Activity of Purified Preparations from *V. alginolyticus*

Enzyme preparation	Subunit	Flavin[b]		Activity[b]	
		FAD	FMN	NQR	NDH
Quinone reductase	α, β, γ	7.2	7.0	112	181
NADH dehydrogenase	β, γ	18.5	3.0	12	255
NADH dehydrogenase	β	21.3	0	0	390
The α fraction	α	0.2	10.3	0	0

[a] nmol/mg protein.
[b] Units/mg protein: NQR, NADH-quinone reductase as measured by the reduction of Q-1 to ubiquinol-1; NDH, NADH dehydrogenase as measured with menadione as the electron acceptor.

membranes by Liponox DCH and was purified by ion-exchange chromatography (Hayashi and Unemoto, 1984, 1986, 1987). The purified NQR-1 is composed of three subunits, α, β, and γ, with apparent M_r of 52, 46, and 32 kDa, respectively, and it contains each subunit in equimolar quantities. Moreover, it contains FAD and FMN in equimolar quantities (Table I). The NADH dehydrogenase was first isolated as the $\beta\gamma$-complex, but further purifications revealed that the β-subunit containing one FAD molecule per molecule corresponds to NADH dehydrogenase. The α-subunit contains FMN as a prosthetic group, but it shows no enzymatic activity by itself. NQR-1 was reconstituted from α- and $\beta\gamma$-subunits, but not from α- and β-subunits, indicating that three subunits are essential for the activity of NQR-1. The role of the γ-subunit is to increase the affinity of the β-subunit for Q-1 (Hayashi and Unemoto, 1987).

The FAD-containing β-subunit reacts with several artificial electron acceptors such as menadione, 2,6-dichlorophenolindophenol, and ferricyanide, but it does not react with Q-5 and MK-4 (Table II). Enzyme activity is stimulated about 5-fold by the addition of either 0.4 M NaCl or KCl, which indicates the salt-dependent activation of the enzyme. However, a specific requirement for Na^+ is not observed in this reaction, and it is also insensitive to HQNO (Hayashi and Unemoto, 1984). The enzyme reacts with Q-1. However, no detectable amount of ubiquinol-1 was produced during the reaction and NADH was consumed in excess of the amount of Q-1 present. Apparently, the enzyme reduces Q-1 by a one-electron transfer pathway, and the semiquinone radicals produced are auto-oxidized by molecular oxygen, forming an oxidation–reduction cycle of the radicals.

On the other hand, the NQR-1 ($\alpha\beta\gamma$-complex) reduces Q-1 to ubiquinol-1 with stoichiometric consumption of NADH, and the reaction stops when all of the Q-1 is converted to ubiquinol-1 (Hayashi and Unemoto, 1984; Unemoto and Hayashi, 1989). The NQR-1 also reduces Q-5 and MK-4 to the respective quinols without the accumulation of free radicals (Table II). It is specifically activated by Na^+ and strongly inhibited by HQNO. Thus, the Na^+-depen-

Table II. Electron Acceptor Specifities of NQR-1, β-Subunit, and NQR-2

Electron acceptor	Concentration (μM)	Relative activity[a]		
		NQR-1	β-Subunit	NQR-2
Q-1	15	100	100	100
Q-5	30	27	0	—
MK-4	10	22	0	—
Menadione	10	120	273	18
	100	214	455	57
DCIP[b]	40	88	392	11
Ferricyanide	1000	149	426	20

[a] Data taken from Hayashi and Unemoto (1984) and Hayashi *et al.*, (1992).
[b] 2,6-Dichlorophenolindophenol.

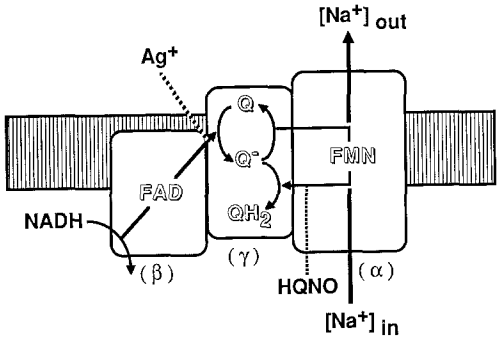

Fig. 1. Electron-transfer pathway, the coupling site of Na^+ translocation, and the sites of Ag^+ and HQNO inhibitions in the Na^+-translocating NQR-1.

dent and HQNO-sensitive sites are not at the reaction catalyzed by the β-subunit but at the reaction catalyzed by the α-subunit. When menadione is used as the electron acceptor, both the β-subunit and NQR-1 produce superoxide radicals, and the NADH is consumed in excess to the amount of menadione. Since the K_m value for menadione is identical with both the enzymes, menadione apparently interacts with the β-subunit of the NQR-1 (Hayashi and Unemoto, 1984).

From these results, the electron transfer pathway from NADH to quinone was formulated as shown in Fig. 1. With respect to the coupling site of Na^+ translocation, proteoliposomes containing purified NQR-1 were prepared by a cholate-deoxycholate dialysis procedure, and the generation of $\Delta\Psi$, positive inside, was monitored by the absorbance band shift of oxonol VI (Hayashi and Unemoto, 1987). By the addition of NADH in the presence of Q-1, the $\Delta\Psi$ is generated concomitant with the formation of ubiquinol-1, which is completely dependent on Na^+ and is strongly inhibited by HQNO. When menadione, which accepts electrons from the β-subunit, is used as the electron acceptor, no detectable magnitude of $\Delta\Psi$ is generated in spite of NADH oxidation. Thus, the Na^+-dependent and HQNO-sensitive reaction catalyzed by the FMN-containing α-subunit directly participates in the generation of $\Delta\Psi$, indicating that this reaction is the coupling site of Na^+ translocation (Fig. 1).

The molecular mass of the protein moiety of NQR-1 is estimated to be 254 kDa in the presence of 0.1% Liponox DCH (Hayashi and Unemoto, 1987). Since this value is twice that of $\alpha + \beta + \gamma$ (130 kDa), the active complex exists as a dimer of $\alpha\beta\gamma$ or $\alpha_2\beta_2\gamma_2$ in 0.1% Liponox solution.

CHARACTERISTIC DIFFERENCE BETWEEN NQR-1 AND NQR-2

As mentioned above, the respiratory chain of *V. alginolyticus* contains two types of NQR. The Na^+-independent NQR-2 was purified from the membranes of a mutant (Nap-1) defective in NQR-1 (Hayashi *et al.*, 1992). In contrast to NQR-1, the purified NQR-2 requires no salt for activity. Electron acceptor specificities are shown in Table II. Inhibitor sensitivities clearly discriminate between NQR-1 and NQR-2. In particular, *p*-chloromercuribenzoate, Ag^+, and HQNO are strong inhibitors to NQR-1. Recent studies on the inhibitory effect of Ag^+ indicate that Ag^+ reacts as a competitive inhibitor for menadione and Q-1 at the β-subunit (unpublished observations). Therefore, the site of Ag^+ inhibition is located on the quinone-binding site of the β-subunit (see Fig. 1). Since HQNO specifically inhibited the Na^+-dependent reaction in the NQR-1, the HQNO inhibition was located on the site of quinol formation at the α-subunit (Fig. 1). Among inhibitors examined, only 5′-AMP inhibited NQR-2 rather than NQR-1 at high concentrations (Hayashi *et al.*, 1992).

Characteristic differences between the NQR-1 and NQR-2 are observed in the mode of quinone reduction and in the energy-transducing capacity. The NQR-2 consumes NADH with the stoichiometric formation of ubiquinol-1 and menadiol, indicating the reduction of Q-1 and menadione by a two-electron transfer pathway (Hayashi *et al.*, 1992). Although the NQR-1 reduced Q-1 to ubiquinol-1 as a whole complex, the formation of menadiol was never observed with the NQR-1. Using inverted membrane vesicles, the NQR-2 was confirmed to have no capacity to generate $\Delta\Psi$ (Smirnova *et al.*, 1990; Kim *et al.*, 1991; Hayashi *et al.*, 1992).

The presence of two types of NQR in the respiratory chain is not unique to *V. alginolyticus*. Yagi (1991) reviewed the bacterial NQR and pointed out the presence of two types of NQR in the respiratory chain of several bacteria. The NQR having an energy coupling site was classified as type-1 NQR (designated NDH-1) and that having no energy coupling site as type-2 NQR (designated NDH-2). The respiratory chain of *Escherichia coli* also has two types of NQR, and the energy-coupled NQR-1 functions as a H^+ pump (Matsushita *et al.*, 1987). The NADH-reacting FMN-containing subunit of NQR-1 was purified from *E. coli* membranes (Hayashi *et al.*, 1989). The natural electron acceptor from this subunit are con-

sidered to be iron-sulfur clusters detected in the membranes (Matsushita *et al.*, 1987; Ohnishi *et al.*, 1987). The purified enzyme primarily reacts with ferricyanide, but menadione is also reduced, with an activity 4.6% of that for ferricyanide. By using a large amount of the FMN-containing subunit, menadione and Q-1 were found to be reduced by a one-electron transfer pathway without the formation of quinols. However, the NQR-2 purified from *E. coli* reduced Q-1 and menadione by a two-electron transfer pathway (Unemoto *et al.*, 1992a).

Another characteristic difference between NQR-1 and NQR-2 from *E. coli* is the fact that the FMN-containing subunit of NQR-1 is very sensitive to NADH preincubation while NQR-2 is not (Hayashi *et al.*, 1989). Therefore, the sensitivity toward NADH preincubation was examined with NQR-1 and NQR-2 from *V. alginolyticus*. It was found that the β-subunit of NQR-1 is very sensitive, whereas the NQR-2 is insensitive, to NADH preincubation (Unemoto *et al.*, 1992a).

MECHANISM OF ENERGY COUPLING

Although the energy-coupled NQR-1 from *E. coli* functions as a H^+ pump and the NQR-1 from *V. alginolyticus* as a Na^+ pump, the NADH-reacting flavoprotein subunit from each bacterium exhibited very similar properties with respect to the mode of quinone reduction and the sensitivity to NADH preincubation. Since each of the NADH- reacting flavoprotein subunit of NQR-1 reduced quinones by a one-electron transfer pathway, the formation of semiquinone radicals as an intermediate is likely to be common to both Na^+ and H^+ pumps. Indeed, a semiquinone EPR signal has been detected in *Thermus thermophilus* HB-8 membranes containing a H^+-translocating NQR-1 (Meinhardt *et al.*, 1990). In contrast, the NQR-2 from each bacterium is insensitive to NADH preincubation and reduces quinone by a two-electron transfer pathway. Thus, the sensitivity of the flavoproteins to NADH preincubation is apparently related to the mode of electron transfer (Unemoto *et al.*, 1992a).

The mechanism of energy coupling at NQR-1 in the respiratory chain remains to be established. The mitochondrial enzyme, known as complex I, contains FMN and at least six iron-sulfur clusters and functions as a H^+ pump. Although the electron-transfer pathway and the site of energy coupling in complex I have not been identified, flavosemiquinone and/or ubisemiquinone radicals were proposed to mediate the redox-linked H^+ translocation (Ragan, 1987). Since NQR-1 from *V. alginolyticus* translocates Na^+ instead of H^+, these semiquinone radicals are unlikely to directly function as a carrier of Na^+. An indirect coupling redox pump mechanism is more plausible for this enzyme. Its subunit structure is relatively simple as compared with that of H^+-translocating NQR-1 from other bacteria and mitochondria. Therefore, the Na^+-translocating NQR-1 must be an excellent model system for the elucidation of the molecular mechanism of coupling between electron transfer and ion translocation.

DISTRIBUTION OF Na^+-TRANSLOCATING NADH-QUINONE REDUCTASE

A variety of Gram-negative marine bacteria such as *V. parahaemolyticus* and other *Vibrio* (Tsuchiya and Shinoda, 1985), psychrophilic *Vibrio* (Takeda *et al.*, 1988), and *Alteromonas*, *Vibrio*, and *Alcaligenes* (Tokuda and Kogure, 1989) have been reported to have a Na^+-translocating NADH-quinone reductase, which is essentially similar to that of *V. alginolyticus* described above. With respect to moderate halophiles, the Na^+-dependent activation of the respiratory chain was first observed with the membranes from *V. costicola* (Unemoto *et al.*, 1977), and later the presence of a respiration-driven Na^+ pump was demonstrated (Udagawa *et al.*, 1986). Moderately halophilic halotolerant Ba_1 was also shown to have Na^+-translocating NADH-quinone reductase (Ken-Dror *et al.*, 1984, 1986a,b). Of seven moderate halophiles from diverse origins, the five Gram-negative bacteria such as *Deleya halophila*, *Halovibrio variabilis*, *Pseudomonas halosaccharolytica*, *Ps. beijerinckii* and an unidentified halophile (NRCC 41227) possess Na^+-translocating NADH-quinone reductase (Unemoto *et al.*, 1992b). Interestingly, the site of the Na^+-dependent reaction is restricted to the NADH-quinone reductase, and other reactions in the respiratory chain show no specific requirement for Na^+ in all these Gram-negative halophiles. In contrast, the respiratory chain of two Gram-positive moderate halophiles, *Marinococcus halophilus* and *Micrococcus varians* subsp. *halophilus*, is not activated by Na^+, and the respiration-driven Na^+ pump is not detected in these organisms. These results strongly suggest that

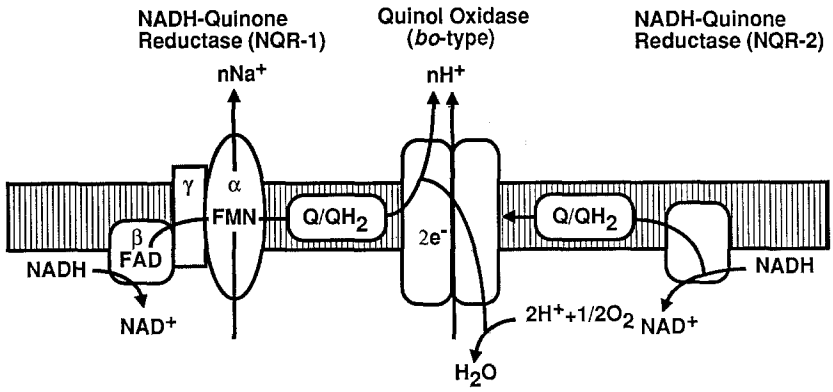

Fig. 2. Respiratory chain of marine *Vibrio*.

the Na^+-translocating NADH-quinone reductase is commonly distributed in Gram-negative marine and moderately halophilic bacteria.

RESPIRATORY CHAIN OF MARINE *VIBRIO*

Recently, cytochrome *bo*-type ubiquinol oxidase was purified from *V. alginolyticus* and its properties were found to be essentially the same as those from *E. coli* (Miyoshi-Akiyama *et al.*, 1992). Since the terminal oxidase of *V. alginolyticus* generates CCCP-sensitive $\Delta\Psi$ (Hayashi *et al.*, 1992), the cytochrome *bo*-type quinol oxidase is considered to function as a H^+ pump. Thus, the marine *Vibrio* has both a redox-driven Na^+ pump and a H^+ pump in its respiratory chain (Fig. 2). The oxidation of NADH via NQR-1 generates both $\Delta\tilde{\mu}_{Na^+}$ and $\Delta\tilde{\mu}_{H^+}$, and the oxidation of NADH via NQR-2 only $\Delta\tilde{\mu}_{H^+}$. These activities may be regulated by the bioenergetic demands of the cells, but the mechanism of regulation remains unsolved.

REDOX-DRIVEN Na^+-PUMP IN OTHER BACTERIA

Surprisingly, Dimroth and Thomer (1989) demonstrated the presence of Na^+-translocating NADH-quinone reductase in an anaerobic bacterium *Klebsiella pneumoniae*, which is known to have a decarboxylase-driven Na^+ pump (Dimroth, 1987). Its physiological role, however, is not clear at present. Semeykina *et al.* (1989) reported the presence of Na^+-motive terminal oxidase in the respiratory chain of halotolerant *Bacillus* sp. strain FTU grown on succinate in the presence of 0.5 M NaCl at pH 8.6. Later, this bacterium was shown to have H^+-motive and Na^+-motive NADH-quinone reductases and terminal oxidases (Kostyrko *et al.*, 1991). The Na^+-motive respiration of this bacterium is induced only if grown at high pH and high NaCl concentrations. The Na^+-motive terminal oxidase was also reported in a nonhalophilic and strictly aerobic bacterium, *Vitreoscilla* sp., where cytochrome *o*-type oxidase is shown to be a redox-driven Na^+ pump (Efiok and Webster, 1990). Avetisyan *et al.* (1989) reported the induction of redox-driven Na^+ pump in *E. coli* grown on succinate at alkaline pH in the presence of NaCl. These growth conditions were selected so as to lower the $\Delta\tilde{\mu}_{H^+}$ such that the cells were expected to be forced to perform a Na^+-motive oxidative phosphorylation (Skulachev, 1989). Recently, Avetisyan *et al.* (1992) reported the involvement of a *d*-type oxidase in the Na^+-motive respiratory chain of *E. coli*. Thus, redox-driven Na^+ pumps are not restricted to marine and moderately halophilic bacteria but are likely to be widely distributed in various species of bacteria. The enzymatic properties of the redox-driven Na^+ pumps described above, however, are not well characterized at present, and further experiments are required to confirm their existence.

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